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(54) Title: PLASMODIUM FALCIPARUM ANTIGENS AND VACCINE AND DIAGNOSTIC USES THEREOF

(54) Titre : ANTIGENES DE PLASMODIUM FALCIPARUM ET LEURS APPLICATIONS VACCINALES ET DIAGNOSTIQUES

(57) Abstract: The invention concerns novel *Plasmodium falciparum* antigens and their vaccine and diagnostic applications. More particularly, the invention concerns immunogenic polynucleotide and polypeptide molecules, compositions comprising them, and methods for diagnosis and vaccination of malaria.

(57) Abrégé : La présente invention se rapporte à de nouveaux antigènes de *Plasmodium falciparum* et à leurs applications vaccinales et diagnostiques. Plus particulièrement, la présente invention vise des molécules polynucléotidiques et polypeptidiques immunogéniques, des compositions les comprenant, et des méthodes de diagnostic et de vaccination du paludisme (malaria).

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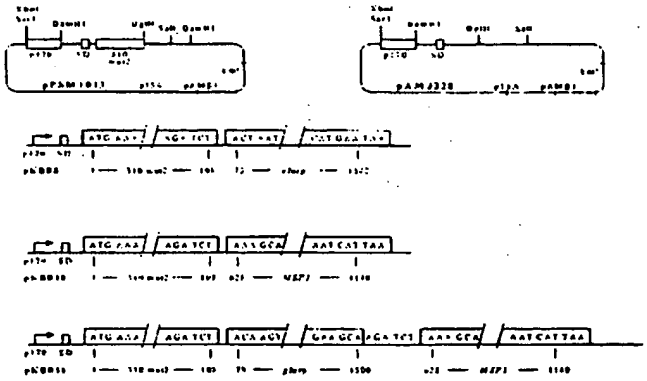

(57) Abstract: A fusion protein, derived from *P. falciparum* Glutamate-rich protein (GLURP) genetically coupled to *P. falciparum* Merozoite surface protein 3 (MSP3) was produced in *Lactococcus lactis* as a secreted recombinant GLURP-MSP3 hybrid protein and experiments showed that the GLURP-part of the hybrid increased the overall antibody response. Immunizations with the hybrid protein consistently generated a stronger antibody response against the individual GLURP and MSP3 domains than a mixture of the two recombinant molecules injected at one site or the individual recombinant molecules injected simultaneously at two different sites. The difference was most pronounced for the MSP3-specific antibody response suggesting that T cell epitopes located in the GLURP RO-region provide help for B-cell epitopes in the MSP3 region. Moreover, when the animals were injected with a mixture of GLURP and MSP3, individual mice tended to mount a predominant antibody response against either molecule: in some animals GLURP was immunodominant whereas in other animals MSP3 was the dominant immunogen. Additionally, the hybrid was also more antigenic than the individual recombinant proteins since the ELISA-titer of naturally occurring IgG antibodies, in clinically immune African adults, against the hybrid protein was higher than the titers against the individual recombinant proteins. The hybrid protein was also demonstrated to be a potential protective antigen as mouse anti-GLURP-MSP3 IgG antibodies were able to inhibit parasite-growth *in vitro* in a monocyte-dependent manner.

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A *Plasmodium falciparum* GLURP–MSP3 chimeric protein; expression in *Lactococcus lactis*, immunogenicity and induction of biologically active antibodies

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Abstract

Plasmodium falciparum malaria is a major cause of morbidity and mortality worldwide. To evaluate the efficacy of a possible vaccine antigen against *P. falciparum* infection, a fusion protein, derived from *P. falciparum* Glutamate-rich protein (GLURP) genetically coupled to *P. falciparum* Merozoite surface protein 3 (MSP3) was produced in *Lactococcus lactis* as a secreted recombinant GLURP–MSP3 fusion protein. The hybrid protein was purified to homogeneity by ion exchange and hydrophobic-interaction chromatography and its composition was verified by MALDI MS, SDS/PAGE and Western blotting with antibodies against antigenic components of GLURP and MSP3. Mice immunized with the hybrid protein produced higher levels of both GLURP- and MSP3-specific antibodies than mice immunized with either GLURP, MSP3 or a mix of both. The protective potential of the hybrid protein was also demonstrated by in vitro parasite-growth inhibition of mouse anti-GLURP–MSP3 IgG antibodies in a monocyte-dependent manner. These results indicate that the GLURP–MSP3 hybrid could be a valuable strategy for future *P. falciparum* vaccine development.

Author Keywords: Author Keywords: *Plasmodium falciparum*; Immunogenicity; Antibodies; *Lactococcus* expression

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A *Plasmodium falciparum* GLURP–MSP3 chimeric protein; expression in *Lactococcus lactis*, immunogenicity and induction of biologically active antibodies

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Abstract

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Keywords: *Plasmodium falciparum*; Immunogenicity; Antibodies; *Lactococcus* expression

1. Introduction

The *Plasmodium falciparum* Glutamate-rich protein (GLURP) and the Merozoite surface protein 3 (MSP3) are both targeted by human IgG antibodies, which can inhibit parasite growth either in vitro in a monocyte-dependent manner [1,2] as well as in vivo by passive transfer in *P. falciparum*-humanized SCID mice [3]. The association of human antibodies against these antigens with clinical protection is also indicated by a number of immuno-epidemiological studies, which demonstrate that the levels of GLURP- and MSP3-specific cytophilic antibodies (IgG1 and IgG3) are significantly associated with a reduced risk of malaria attacks [4–6]. The major B-cell epitopes recognized by these human IgG antibodies have been localized to conserved sequences in the $GLURP^{T1-489}$ and $MSP3^{212-257}$ regions, respectively [1,6–9].

Since vaccines based on GLURP and MSP3 aim at inducing the same type of immune responses, i.e. high levels of cytophilic antibodies, we decided to produce the respective $GLURP^{25-500}$ and $MSP3^{212-363}$ regions together as a recombinant hybrid-protein in *Lactococcus lactis*. This hybrid offers the possibility to investigate the vaccine potential of both antigens in single immunizations and can potentially increase the immunogenicity by combining a wider range of B and T helper cell epitopes. Different regions of these antigens have previously been produced in *Escherichia coli* fused to various affinity-tags [10–12]. Whereas such additional sequences are advantageous for purification they also pose a potential problem because host immune responses may be biased by foreign sequences. It is, therefore, desirable to explore expression systems, which aim to produce the recombinant protein without a vector-encoded affinity-tag. *L. lactis* was chosen as expression host because (i) it is a well characterized industrial microorganism, generally recognized as safe (GRAS), best known for its use in the production of fermented dairy products, (ii) it can be

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REVIEW

Malaria Vaccine: Candidate Antigens, Mechanisms, Constraints and Prospects

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More than 30 years after the first report of successful vaccination against malaria using radiation-attenuated sporozoites, an effective malaria vaccine is not yet available. However, field and experimental data indicate that it can be developed. An astonishing amount of data has accumulated concerning parasite biology, host–parasite interactions, immunity and escape mechanisms, targets and modulators of immune responses. Nevertheless, so far this knowledge has not been enough to make us understand how to properly manipulate the whole system to build an effective vaccine. In this article, we describe candidate antigens, mechanisms, targets and trials performed with potential malaria vaccines and discuss the approaches, *in vivo* and *in vitro* models, constraints and how technologies such as DNA vaccination, genomics/proteomics and reverse immunogenetics are providing exciting results and opening new doors to make malaria vaccine a reality.

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THE MALARIA PROBLEM

Mankind has stepped into the 21st century and diseases such as malaria still represent a major threat to populations in many parts of the world. The exact extent of the malaria problem is not known, but several estimates provide a gloomy picture of the situation [1]. It is estimated that between 400 and 900 million febrile episodes occur every year only in African children, with a minimum of 750,000 deaths (probably up to 3 million). In addition, nonsevere cases yet cause considerable morbidity in acute or chronic disease, with serious socio-economical consequences. Sub-Saharan Africa is the most affected region in the world, but malaria is also a serious problem in several other places, such as South-East Asia, Oceania, Middle East and Latin America. Historically, in the late 1940s, there was great optimism in the fight against malaria, mainly owing to the introduction of dichlorodiphenyltrichloroethane (DDT) for vector control and of chloroquine as a very efficient antimalarial drug. These and other available control tools prompted the World Health Organization (WHO) to launch a campaign for complete malaria eradication. The campaign was very successful in places such as Mediterranean countries and even many regions in the tropics. In Brazil, for instance, the number of cases per year dropped from nearly 6 million in the 1940s to around 37,000 in 1962 [2] and became restricted to the Amazon region. But since then, malaria has seen resurgence and/or is spreading in many areas; in the Amazon region of Brazil the number of cases per year increased from nearly 37,000 in 1962 to around 600,000 in the late 1990s. Indeed, already in the 1960s it became clear that eradication was not feasible, and the WHO strategy was switched, aiming to control rather than to eradicate. Environmental conditions, population habits and living conditions, migratory movements of people to endemic areas, regional development projects, resistance of parasites to drugs and of mosquitoes to DDT, among other factors, greatly favoured the maintenance of malaria in the endemic regions. All the difficulties concerning malaria control justify the search and adoption of new tools and measures to minimize the impact of malaria on the

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DEVELOPMENT OF A HETEROLOGOUS GENE EXPRESSION SYSTEM FOR USE IN *LACTOCOCCUS LACTIS*

A Novel Gram-positive Expression System

Key words: *Lactococcus lactis*, lactic acid bacteria, Gram-positive, heterologous gene expression, recombinant proteins, P170 promoter, secretion, vaccine antigens.

Abstract. We have developed a gene expression system for use in the lactic acid bacterium *Lactococcus lactis*. The expression is controlled by the *L. lactis* promoter, P170, which is up-regulated by low-pH during the transition to stationary phase. The P170 promoter is coupled to a modified *L. lactis* signal peptide, SP310mut2, to mediate secretion of the recombinant protein. Optimisation of genetic components, growth conditions and medium composition has resulted in the production of approximately 300 mg of secreted protein per litre culture. We have successfully produced a number of heterologous proteins from both prokaryotic and eukaryotic organisms. The system is currently used for the production of bacterial vaccine components for use in humans.

1. INTRODUCTION

Lactic acid bacteria have been used for centuries for the manufacturing of fermented foods and are therefore Generally Recognised As Safe (GRAS) organisms. They are acid tolerant and acidify their own environment by converting fermentable carbohydrates, e.g. lactose or glucose, primarily to lactic acid. All lactic acid bacteria are grouped within the Gram-positive eubacteria.

The outer cell membrane of many Gram-negative bacteria, e.g. *E. coli*, contains lipopolysaccharides, LPS, which are pyrogenic in humans and other mammals. The LPS are generally referred to as endotoxins. The presence of endotoxins in the production of recombinant proteins in Gram-negative bacteria poses considerable difficulties during product purification (for review, see Petsch and Anspach, 2000). Gram-positive bacteria do not have an outer cell membrane and do not produce endotoxins. Like most other Gram-positive bacteria, *Lactococcus lactis* is capable of secreting proteins to the extracellular environment. This is in contrast to Gram-negative bacteria in which the majority of secreted proteins is destined for the periplasmic space. *L. lactis* secretes a small number of proteins. Consequently, the supernatants into which the heterologous proteins are secreted are largely free of

O-W. Merten et al. (eds.).
Recombinant Protein Production with Prokaryotic and Eukaryotic Cells, 269–275.

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The Glutamate-Rich Protein (GLURP) of *Plasmodium falciparum* Is a Target for Antibody-Dependent Monocyte-Mediated Inhibition of Parasite Growth In Vitro

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Monocyte-dependent as well as direct inhibitory effects of antimalarial antibodies point toward antigens accessible at the time of merozoite release as targets for biologically active antibodies capable of mediating protection against *Plasmodium falciparum*. The glutamate-rich protein (GLURP), being an antigen associated with mature schizont-infected erythrocytes, was therefore the object of the present investigation, in which we analyzed whether anti-GLURP antibodies can either interfere directly with merozoite invasion or act indirectly by promoting a monocyte-dependent growth inhibition, antibody-dependent cellular inhibition. GLURP-specific human immunoglobulin G (IgG) antibodies, from pooled IgG of healthy Liberian adults who were clinically immune to malaria, were purified by affinity chromatography on columns containing R0 (N-terminal nonrepetitive region of GLURP) or R2 (C-terminal repetitive region of GLURP) recombinant protein or synthetic peptides as ligands. Analysis of the pattern of reactivity of highly purified anti-GLURP antibodies led to the definition of at least four B-cell epitopes. One epitope was specific for R0, two were specific for R2, and the fourth displayed cross-reactivity between R0 and R2. None of the purified IgG antibodies had direct invasion-inhibitory effects, even at high concentrations. In contrast, when allowed to cooperate with monocytes, all anti-GLURP IgG preparations mediated a strong monocyte-dependent parasite growth inhibition in a dose-dependent manner.

Epidemiological surveys performed in areas of intense malaria transmission have consistently shown that individuals who are continuously exposed to repeated malaria infection gradually develop clinical immunity (14, 20, 29). This acquired immunity is strong, although incomplete, and is nonsterilizing (3, 25, 26). Experiments with antibodies purified from the sera of African adults who were clinically immune to malaria and given by passive transfer to susceptible children have established that immunoglobulin G (IgG) is at least a main component of defense against the asexual blood stage of *Plasmodium falciparum* (5, 9, 11). Recent passive transfer experiments have enabled us to acquire clinically demonstrated protective antibodies from the donor and nonprotective antibodies from the recipients. These sets of antibodies were used to assess the extent to which the in vitro data correlated with the in vivo results for each recipient isolate (5). Results from these in vitro studies suggested that clinically protective antibodies had little direct effect on merozoite invasion, but that they could act in conjunction with blood monocytes to contain parasite multiplication. This mechanism was called antibody-dependent cellular inhibition (ADCI) (5, 17, 19).

The assay provides a screen to select molecules which may be targeted by clinically effective antibodies. Further experiments have indicated that antibody-monocyte cooperation in parasite inhibition is mediated not through parasite opsonization but rather through indirect effects. These activities were mediated by soluble monocyte-derived substances whose release was triggered through monocyte interaction with cytophilic antibodies bound to merozoite antigens (7). A critical role for merozoite surface molecules in this mechanism is also supported by the identification of Msp3, a new molecule from the merozoite surface, when an expression library was screened by ADCI (23).

Based on the published immunoepidemiological data for the glutamate-rich protein (GLURP) (4, 12, 13, 15) and the report that this molecule is located on the surface of the merozoite (2), we chose to investigate the potential of affinity purified anti-GLURP human IgG in assays of direct parasite inhibition and to compare this with activity in ADCI assays.

MATERIALS AND METHODS

Antigens. The two recombinant GLURP fragments, GLURP$_{R0-1409}$ (R0), and GLURP$_{R2-1178}$ (R2), were purified as previously described (27). The five peptides, GL8 (GGPKLRGNVTSNIKFPSDNKGK [amino acids (aa) 36 to 57]), GL6 (KQNSQIPSLDLKEPTNEDI [aa 309 to 327]), GL9 (PNFVDSQPNPQE PVEPSFVKJEKVPSEEN [aa 732 to 760]), GL5 (EFKEJNEDDKSAHIQHE IVEVEEJLPEDD [aa 853 to 881]), and GL7 (KNKKKSSFITYISTKKFK [aa 1215 to 1232]) correspond to repetitive as well as nonrepetitive regions of GLURP. The peptides MSP-3b and RESA have been described previously (23). Synthetic peptides were produced according to standard peptide synthesis procedures (22). Cleavage and deblocking used trifluoromethanesulfonic acid followed by an ether wash.

Immunoprecipitation. Metabolic labelling and immunoprecipitation of *P. falciparum* polypeptides were performed as described previously (27). Immunoprecipitations were performed as follows. Affinity-purified human anti-R0 and anti-R2 antibodies (fractions 1 and 2) were added to 0.5 ml of whole-cell lysate or to 250 μl of culture supernatant plus 250 μl of 2× radioimmunoprecipitation assay (RIPA) buffer (1× RIPA buffer is 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% sodium dodecyl sulfate [SDS]), and the mixtures were incubated overnight at 4°C with rotation. Seventy-five microliters of a 50% suspension of protein A-Sepharose beads (Pharmacia,

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Association between Protection against Clinical Malaria and Antibodies to Merozoite Surface Antigens in an Area of Hyperendemicity in Myanmar: Complementarity between Responses to Merozoite Surface Protein 3 and the 220-Kilodalton Glutamate-Rich Protein

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We performed a longitudinal clinical and parasitological follow-up study in OoDo, a village in southeast Asia in which malaria is hyperendemic, in order to assess the association between protection against malaria attacks and antibodies to three currently evaluated vaccine candidates, merozoite surface protein 1 (MSP1), MSP3, and the 220-kDa glutamate-rich protein (GLURP) from *Plasmodium falciparum*. Our results showed that the levels of cytophilic immunoglobulin G3 (IgG3) antibodies against conserved regions of MSP3 and GLURP were significantly correlated with protection against clinical *P. falciparum* malaria. In contrast, the levels of noncytophilic IgG4 antibodies against GLURP increased with the number of malaria attacks. Furthermore, we observed a complementary effect of the MSP3- and GLURP-specific IgG3 antibodies in relation to malaria protection. In the individuals that did not respond to one of the antigens, a strong response to the other antigen was consistently detected and was associated with protection, suggesting that induction of antibodies against both MSP3 and GLURP could be important for the development of protective immunity. The complementarity of the responses to the two main targets of antibody-dependent cellular inhibition identified to date provides the first rational basis for combining these two antigens in a hybrid vaccine formulation.

In regions where malaria is hyperendemic, adults develop potent but nonsterile immunity against malaria in which individuals chronically harbor low-grade parasitemia and only occasionally suffer from mild clinical malaria, a clinical state known as premunition (12, 24). Antibodies have been repeatedly shown to play an important role in the development of premunition (20, 26), and numerous immunological studies have suggested that human antibodies of the cytophilic subclasses (immunoglobulin G1 [IgG1] and IgG3) are particularly critical in this respect (2, 5, 6, 18, 21, 27, 28, 30). This antiparasite immunity is a strain-independent, nonsterilizing type of immunity which is acquired after lengthy exposure (ca. 15 to 20 years) to the parasite. It is commonly observed in Africa and in some parts of Papua New Guinea, but it has only recently been documented in southeast Asia (29). Although antibodies can act directly upon merozoite invasion of red blood cells (reviewed in reference 4), the most efficient in vivo mechanism for antibody-mediated parasite control in areas where the disease is endemic requires the participation of monocytes (16, 17). The antibody-dependent cellular inhibition (ADCI) assay mimics this cooperation between monocytes and cytophilic parasite-specific antibodies, and ADCI appears today to be the best in vitro surrogate marker of acquired immunity against *Plasmodium falciparum* blood stages (11). Two molecules have been identified as targets of human antibodies that are effective in ADCI; these molecules are the 48-kDa merozoite surface protein 3 (MSP3) (22) and the 220-kDa glutamate-rich protein (GLURP) (31). Several immunoepidemiological studies performed in geographically separated areas of Africa have demonstrated that high levels of GLURP- and MSP3-specific cytophilic antibodies are significant predictors of protection against clinical malaria (10, 23), providing epidemiological support for the concept that antibodies against GLURP and MSP3 can actively control parasite multiplication in vivo by cooperation with cells bearing FcγII receptors (6). These receptors exhibit higher affinity for the IgG3 subclass than for the IgG1 subclass (25). The B-cell epitopes recognized by these human antibodies have been mapped within both the GLURP and MSP3 molecules, and nucleotide sequencing has demonstrated that these important epitopes are highly conserved in a number of *P. falciparum* laboratory lines and field isolates from Africa and Asia (8, 15, 19). In view of the conservation of the epitopes, we investigated whether cytophilic antibodies against GLURP and MSP3 are involved in the development of immunity to clinical malaria in an Asian population in Myanmar, as they have been reported to be in Africa (i.e., in a different human and parasite genetic background). Since numerous reports have argued that antibodies against the C terminus of

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DIO

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Immunization of *Saimiri sciureus* Monkeys with *Plasmodium falciparum* Merozoite Surface Protein-3 and Glutamate-Rich Protein Suggests that Protection is Related to Antibody Levels

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Abstract

The immunogenicity and protective efficacy of various antigen-adjuvant formulations derived either from the merozoite-surface protein-3 (MSP-3) or the glutamate-rich protein (GLURP) of *Plasmodium falciparum* were evaluated in *Saimiri sciureus* monkeys. These proteins were selected for immunogenicity studies based primarily on their capacity of inducing an antibody-dependent cellular inhibition effect on parasite growth. Some of the *S. sciureus* monkeys immunized with MSP-$3_{212-380}$-AS02 or GLURP$_{27-500}$-alum were able to fully or partially control parasitaemia upon an experimental *P. falciparum* [Falciparum Uganda Palo Alto (FUP-SP) strain] blood-stage infection, and this protection was related to the prechallenge antibody titres induced. The data are indicative that MSP-3 and GLURP can induce protective immunity against an experimental *P. falciparum* infection using adjuvants that are acceptable for human use and this should trigger further studies with those new antigens.

Introduction

The role of antibodies in the immunity against the blood stages of *Plasmodium falciparum* malaria has been well established by passive transfer experiments performed by Cohen and McGregor [1] and later by Bouharoun-Tayoun and coworkers [2]. In the latter, immunoglobulins (Igs) obtained from malaria immune African individuals caused striking decrease in parasitaemia when passively transferred to nonimmune Thai patients. The same Ig preparations active *in vivo* were unable to directly inhibit parasite growth *in vitro* but showed a strong inhibitory effect when allowed to cooperate with human monocytes in an antibody-dependent cellular inhibition (ADCI) fashion. The ADCI assay has thus been considered a good *in vitro* correlate of antimalaria immunity acting *in vivo* [3] and has since been used to screen for potential malaria vaccine candidate antigens. Antibodies against merozoite-surface protein-3 (MSP-3) [4] and glutamate-rich protein (GLURP) [5] strongly inhibit *P. falciparum* growth in ADCI assays. Evaluation of the fine specificity of affinity purified anti-MSP-3 and anti-GLURP antibodies revealed that those directed against the MSP-3b and P3 epitopes mediated the strongest ADCI effects [4, 6]. Immuno-epidemiological studies have demonstrated that high levels of cytophilic MSP-3- and GLURP-specific antibodies are significantly associated with protection against *P. falciparum* malaria [4, 7, 8]. The B-cell epitopes that are targeted by these human antibodies are highly conserved among isolates from different geographical origins [9, 10], suggesting that they are functionally constrained and not subjected to selection for variation. Such characteristics support the further evaluation of MSP-3 and GLURP as malaria vaccine candidate antigens. In the present work, we have proceeded to a preliminary preclinical evaluation of several constructs derived from MSP-3 and GLURP in combination with different adjuvants in the New World primate *Saimiri sciureus*, which is together with Aotus [11, 12], one of the WHO-recommended

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IM-95 – IMMUNIZATION OF *SAIMIRI SCIUREUS* MONKEYS WITH MSP-3 AND GLURP, TWO *PLASMODIUM FALCIPARUM* ANTIGENS TARGETS OF PROTECTIVE ANTIBODIES

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Merozoite Surface Protein-3 (MSP-3) and Glutamate Rich Protein (GLURP) of *P. falciparum* are considered malaria vaccine candidates due to characteristics such as wide recognition by human immune sera and ability of antibodies against them to inhibit *P. falciparum* growth *in vitro* in Antibody Dependent Cellular Inhibition (ADCI) assays. The objective of this work is to perform pre-clinical evaluation of constructed antigens derived from MSP-3 and GLURP in combination with several adjuvants in the *Saimiri sciureus* monkey, one of the W.H.O.-recommended non-human primate models for studies on malaria.

Five antigen-adjuvant combinations have been used: a) MSP-3: DG210-Incomplet Freund's Adjuvant (IFA) and MSP-3 C-terminus in association with: Ribi, Montanide or SBAS2; b) GLURP: R0-alum [$Al(OH)_3$]. Each group received 4 or 5 shots in a time interval of 6 to 10 months. Immune response has been analyzed by ELISA, immunofluorescence (IFAT), immunoblotting and ADCI.

Results obtained so far have shown that all antigen-adjuvant combinations are able to elicit high serum titres of antibodies directed against the immunogen, as detected in ELISA. However, the same sera showed very poor or no recognition of the parasite in IFAT or immunoblot assays; in addition, they were not able to inhibit *P. falciparum* growth in ADCI, whereas IgG or anti-MSP3 antibodies purified from saimiri immune sera showed strong effect.

We currently perform deeper immunological analysis of sera from immunized animals (cytokine dosage, ADCI, IFAT, immunoblot); data will be compared with similar analysis performed with sera and purified antibodies from saimiris hyperimmunized by repeated infections, which may lead to the optimization of formulations to be used in further studies.

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

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PCT/EP2004/012910	22/10/2004	24/10/2003
Applicant		
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1. **Basis of the report**
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In vivo veritas: lessons from immunoglobulin-transfer experiments in malaria patients

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In most fields of medicine, experimentation starts with studies *in vitro*, moves to animal models and eventually proceeds to research on humans. Malaria provides a good example of the limits of this progression. The most important malarial parasite of man, *Plasmodium falciparum*, only infects man. The specificity of this relationship accounts for the many differences which exist between artificial models of falciparum malaria and natural infections. Ultimately, human infections appear to be the sole, relevant 'model' for the study of human–*Plasmodium* interactions. Immunoglobulin-transfer experiments, for example, clearly indicated that antibodies mediated the state of acquired immunity called premunition. However, further studies *in vitro* or in animal models led to conflicting results about how the antibodies acted. Transfer experiments in human volunteers, appropriately coupled to in-vitro studies, seemed the only way to help solve this issue. The design of these investigations, with its technical and ethical aspects, is reviewed here, along with the main published and unpublished results. The identification of a monocyte-mediated, antibody-dependent (ADCI) mechanism led to a new merozoite-surface antigen (MSP-3) being identified and provided an explanation for the long delay in the acquisition of protection. It appears that experiments in humans not only help to confirm indications obtained using animal models, but can also have a truly exploratory role, since they can both raise completely new issues and provide answers to them.

Several years ago, what was then considered to be a somewhat odd and old-fashioned experiment was performed (Sabchareon *et al.*, 1991). This reproduced earlier, passive transfers of immunoglobulin (IgG) in subjects infected with *Plasmodium falciparum* (Cohen *et al.*, 1961; McGregor *et al.*, 1963). It was hoped that this and similar experiments, if appropriately coupled to in-vitro studies, would help in identifying the mechanisms underlying naturally acquired, protective immunity against *P. falciparum*. The experiment was also intended to be the first step in a novel approach to vaccine design, which would rely solely on the study of human–*Plasmodium* interactions and not on infections in animal models. One critical aspect of this exacting study was to define the ethically acceptable conditions, in accordance with current regulations, for limited experiments with passive transfer of human antibodies from immune individuals to patients with *P. falciparum* infections. The rationale and constraints of this approach, and the new knowledge that it brought, are reviewed below.

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A Novel Merozoite Surface Antigen of *Plasmodium falciparum* (MSP-3) Identified by Cellular-Antibody Cooperative Mechanism Antigenicity and Biological Activitiy of Antibodies

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We report the identification of a 48kDa antigen targeted by antibodies which inhibit Plasmodium falciparum *in vitro growth by cooperation with blood monocytes in an ADCI assay correlated to the naturally acquired protection. This protein is located on the surface of the merozoite stage of* P. falciparum, *and is detectable in all isolates tested. Epidemiological studies demonstrated that peptides derived from the amino acid sequence of MSP-3 contain potent B and T-cell epitopes recognized by a majority of individuals living in endemic areas. Moreover human antibodies either purified on the recombinant protein, or on the synthetic peptide MSP-3b, as well as antibodies raised in mice, were all found to promote parasite killing mediated by monocytes.*

Key words: malaria - *Plasmodium falciparum* - merozoite - MSP-3

As a means to select for molecules capable of inducing protective immunity to malaria, we have chosen to rely on those mechanisms mediating premunition, a naturally-occurring type of protection, which is known to be acquired progressively by individuals repeatedly exposed to *Plasmodium falciparum*. We have shown previously that antibodies from protected adults had no major effect upon parasite invasion and growth in red blood cells, but rather acted indirectly by an antibody-dependent cellular inhibition (ADCI) effect mediated by blood monocytes (Druilhe & Bouharoun-Tayoun 1992). The relevance of ADCI to clinical protection was confirmed by passively transferring immunity in humans by means of IgG (Sabchareon et al. 1991) and by performing *in vitro* correlations with the biological material collected during the passive transfer (Bouharoun-Tayoun et al. 1990). Since this indirect effect of monocytes depends on the ability to raise antibodies which are cytophilic to monocytes, the isotype distribution of antimalarial antibodies is critical. It was shown that non-cytophilic IgG2 and / or IgM classes predominated in the various groups of non-protected subjects, whilst cytophilic IgG3 and also IgG1 classes were the main isotypes produced by protected individuals (Bouharoun-Tayoun & Druilhe 1992).

On the basis of the malaria polypeptides recognized by cytophilic/non-cytophilic antibodies and the results of *in vitro* screening with the ADCI assay, we identified a merozoite surface protein, of 48 kDa molecular weight, and characterized its B and T epitopes.

RESULTS

IDENTIFICATION OF THE 48 KDA PROTEIN

One monoclonal antibody of IgM class which we produced previously (Soulier et al. 1982) was the only one found among many studied, able to block the ADCI effect mediated by protected adult IgG. When used to screen a *P. falciparum* DNA library (Guerin-Marchand et al. 1987), this mAb did not label any of the clones tested. Since the mAb 245 was able to show a positive result with native proteins by IFA and to compete with other antibodies in the recognition of native proteins in bio-assays (ADCI), it was decided to screen a DNA library by competition assays. The recombinant proteins from a subset of 100 clones were used in a

Brief Definitive Report

Human Malaria in Immunocompromised Mice: An In Vivo Model to Study Defense Mechanisms against *Plasmodium falciparum*

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Abstract

We have recently described that sustained *Plasmodium falciparum* growth could be obtained in immunodeficient mice. We now report the potential of this new mouse model by assaying the effect of the passive transfer of antibodies (Abs) which in humans have had a well-established effect.

Our results show that the total African adult hyperimmune immunoglobulin Gs (HI-IgGs) strongly reduce *P. falciparum* parasitemia similarly to that reported in humans, but only when mice are concomitantly reconstituted with human monocytes (HuMNs). In contrast, neither HI-IgGs nor HuMNs alone had any direct effect upon parasitemia. We assessed the in vivo effect of epitope-specific human Abs affinity-purified on peptides derived either from the ring erythrocyte surface antigen (RESA) or the merozoite surface protein 3 (MSP3). The inoculation of low concentrations of anti-synthetic peptide from MSP3, but not of anti-RESA Abs, consistently suppressed *P. falciparum* in the presence of HuMNs. Parasitemia decrease was stronger and faster than that observed using HI-IgGs and as fast as that induced by chloroquine. Our observations demonstrate that this mouse model is of great value to evaluate the protective effect of different Abs with distinct specificity in the same animal, a step hardly accessible and therefore never performed before in humans.

Key words: mouse model • protective immunity • antibody-dependent cellular inhibition • merozoite surface protein 3 • ring erythrocyte surface antigen

Introduction

Malaria due to *Plasmodium falciparum* causes the highest mortality in individuals living in endemic countries. The development of weapons, particularly vaccines, to fight this major public health problem has been severely hampered by the lack of a suitable in vivo model. Only a very small number of primate species are receptive to *P. falciparum*, which scarcity and cost preclude any widespread use (1–3).

Animal models for falciparum malaria in immunodeficient, chimerical mice have been described recently (4–6). We used mice bearing the mutations beige, X-linked immunodeficient, and nude (BXN) affecting the T and B cell functions. In this model, the partial depletion of circulating and tissue phagocytes was crucial to the successful grafting of human red blood cells (HuRBCs) and to the development of the erythrocytic cycle of *P. falciparum* (4). After our initial report, further progress was made in controlling mouse macrophages which resulted in obtaining consistently long-lasting, stable parasitemias. In the BXN mice, the morphology of the *P. falciparum* parasite and the responses to currently employed antimalarial drugs were similar to those observed in humans (Moreno, A., E. Badell, N. van Rooijen, and P. Druilhe, manuscript submitted for publication), therefore confirming the validity of this in vivo *P. falciparum* model.

In view of the need to improve the understanding of host–parasite interactions for vaccine development, we evaluated the relevance of this *P. falciparum* mouse model for the analysis of the effects of different components of the human immune system on the parasite. There is considerable in vivo evidence that the IgG constitutes an efficient protective arm of the immune system against *P. falciparum* erythrocytic stages in humans (7–9). Therefore, passive

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Brief Definitive Report

A Single Fragment of a Malaria Merozoite Surface Protein Remains on the Parasite During Red Cell Invasion and Is the Target of Invasion-inhibiting Antibodies

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Summary

A complex of polypeptides derived from a precursor is present on the surface of the malaria merozoite. During erythrocyte invasion only a small fragment from this complex is retained on the parasite surface and carried into the newly infected red cell. Antibodies to this fragment will interrupt invasion.

Proteins on the surface of malaria merozoites are targets of an immune response and are candidate vaccine components. The precursor to major merozoite surface antigens, referred to here as MSP1 (merozoite surface protein 1) is synthesized during schizogony and is present on the merozoite as a complex of fragments derived by proteolytic processing (1, 2). An 83-kD fragment, $MSP1_{83}$ can be isolated from culture supernatants, and mAbs specific for $MSP1_{83}$ do not react with newly invaded (ring-stage) parasitized erythrocytes in an immunofluorescence (IF) assay (2–3), suggesting that this part of the complex is shed during the invasion process. A second group of MSP1-mAb do react in IF with ring forms, and all appear to react with reduction-sensitive epitopes on a COOH-terminal 42–45-kD fragment ($MSP1_{42}$) and a 16–19-kD polypeptide subfragment ($MSP1_{19}$) from the cysteine-rich COOH terminus of the precursor (2, 4–6). There is evidence that a MSP1-specific immune response can protect against blood-stage challenge (1, 7), and certain mAbs to either the *Plasmodium falciparum* MSP1 or homologues in other species have been shown to inhibit the growth of the parasite in vitro (8–9) or protect on passive transfer in vivo (10–12). The function of MSP1 is unknown, although it may be involved in red cell invasion, possibly in a receptor role (13).

Here we demonstrate that MSP_{19} is specifically carried into the red cell and that antibodies to this fragment can inhibit invasion. These observations suggest that processing must take place for merozoite invasion to proceed.

Materials and Methods

Antibodies. MSP1-specific mAbs were used: 12.1 and 13.2 (anti-$MSP1_{83}$); 13.1 and X509 (anti-$MSP1_{42}$ N-terminus); 7.5, 12.8, and 12.10 (anti-$MSP1_{42}$ COOH terminus and $MSP1_{19}$) (2). mAb 13.4 (anti-MSP2, merozoite surface protein 2, 46-kD antigen) and 13.3 (anti-lactate dehydrogenase) were used as controls (14). X509 is a product of a human EBV-transformed cell line derived from a Gambian donor (Blackman, M.J., et al., manuscript in preparation).

Parasites. *P. falciparum*, strain FCB-1, and clones T9/94 and T9/96 were maintained essentially as described previously (3), and synchronized by a combination of sorbitol and Percoll treatments when appropriate.

Immunoelectron Microscopy. *P. falciparum* (FCB-1 strain) cultures ~1 h after invasion and containing 50–60% ring forms were fixed, cryosectioned and incubated with ascitic fluid containing the mAbs. Antibody binding was revealed by incubation with rabbit anti-mouse Ig and protein A–gold. After washing in PBS, sections were stained with 2% uranyl acetate and viewed in a JEOL 100B electron microscope (15).

Production of Merozoites and Saponin-lysed Rings. Merozoites were harvested at 1–1.5 h intervals from cultures enriched for schizonts (85–90% parasitaemia at a hematocrit of ~0.2%) and washed in PBS containing the following protease inhibitors: 1 mM PMSF, 2 mM tosyl-L-lysine chloromethyl ketone (TLCK), 0.1 mM tosyl-L-phenylalanine chloromethyl ketone (TPCK), and chymostatin, leupeptin, antipain, and aprotinin (all at 10 μg/ml).

To prepare ring-stages, schizonts were cultured in the presence of fresh, washed erythrocytes at 2% hematocrit (15–20% parasitaemia). After 7 h the cells were centrifuged twice over 67.5% isotonic Percoll to remove any remaining schizonts. The resulting pellets containing only uninfected erythrocytes and ring-stage parasites (with <0.01% schizont contamination) were washed twice in serum-free medium and resuspended in ice-cold PBS containing protease inhibitors (PBS/PI). Saponin (10% wt/vol in PBS, BDH Ltd., Poole, UK) was added slowly with gentle mixing to a final concentration of 0.005%, and the suspension was incubated on ice until lysis began. An equal volume of PBS/PI was added, and

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Mechanisms Underlying the Monocyte-mediated Antibody-dependent Killing of *Plasmodium falciparum* Asexual Blood Stages

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Summary

The relevance of the antibody-dependent cellular inhibition (ADCI) of *Plasmodium falciparum* to clinical protection has been previously established by in vitro studies of material obtained during passive transfer of protection by immunoglobulin G in humans. We here report further in vitro investigations aimed at elucidating the mechanisms underlying this ADCI effect. Results obtained so far suggest that (*a*) merozoite uptake by monocytes (MN) as well as by polymorphonuclear cells has little influence on the course of parasitemia; (*b*) the ADCI effect is mediated by a soluble factor released by MN; (*c*) this or these factors are able to block the division of surrounding intraerythrocytic parasites at the one nucleus stage; (*d*) the critical triggering antigen(s) targeted by effective Abs would appear to be associated with the surface of merozoites, as opposed to that of infected red blood cells; (*e*) the MN receptor for Abs effective in ADCI is apparently FcγRII, and not RI; (*f*) MN function is up- and down-regulated by interferon-γ and interleukin 4, respectively; and (*g*) of several potential mediators released by MN, only tumor necrosis factor (TNF) proved of relevance. The involvement of TNF in defense may explain the recently described increased frequency of the TNF-2 high-expression promoter in individuals living in endemic regions despite its compromising role in severe malaria.

The asexual blood stages (ABS)[1] of malaria parasites are the only stages responsible for the pathology of the disease. They are also the most accessible to investigation, because they are the only ones whose cultivation is easy. These stages have therefore been the subject of most of the studies of malaria aimed at understanding the basis of protective immunity for vaccination purposes. Based on passive transfer of Abs, there is considerable in vivo evidence that IgG constitutes an efficient arm of the immune system against erythrocytic stages in humans infected with *Plasmodium falciparum* (1–3), in South American monkeys infected with *P. falciparum* (4), and in a number of primate and rodent models (for review see reference 5). How these Abs act upon ABS, however, is far less clear. A number of hypotheses or beliefs have dominated the past decade, among which the inhibition of merozoite invasion is the most popular, together with a number of alternative hypotheses such as the inhibition of cytoadherence (thought to lead to the destruction of schizonts in the spleen), the inhibition of schizont-infected RBC (SIRBC) rosetting, the inhibition of merozoite dispersal, and opsonization of SIRBC.

Our initial observation that Abs from individuals with acquired protective immunity against ABS had no direct antiparasite effect in vitro led us to investigate other modes of action. We reported an Ab-dependent cellular cytotoxicity (ADCC)-like effect exerted by blood monocytes (MN), which was named Ab-dependent cellular inhibition (ADCI) to reflect the fact that the readout was an inhibition of parasite growth. The relevance of the MN-mediated ADCI observed in vitro was further validated by close in vivo/in vitro correlations: A strong ADCI effect could be elicited only by protective Abs, i.e., Abs whose clinical effect had been demonstrated by passive transfer in humans. Conversely, the preexisting Abs that proved clinically ineffective in the same individuals upon the same strains did not promote ADCI (6). The concept of Abs being effective by cooperation with effector cells was further supported by isotype studies showing a clear correlation between the ratio of cytophilic/noncytophilic Abs and the clinical status of protection (7).

In parallel with studies aimed at using this assay to identify protective antigen(s) (8), we have focused on the study of the ADCI mechanism itself. The present report summa-

[1] *Abbreviations used in this paper:* ABS, asexual blood stage; ADCC, antibody-dependent cellular cytotoxicity; ADCI, antibody-dependent cellular inhibition; BHA, butylated hydroxyanisole; CL, chemiluminescence; CLI, CL index; DCLI, delta CLI; FOR, free oxygen radical; HI-IgG, hyperimmune IgG; L-NMMA, N^G-monomethyl-L-arginine; MLI, maximum light intensity; MN, monocyte; N-IgG, control IgG; SIRBC, schizont-infected RBC; SGI, specific growth inhibitory index.

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Levels of Antibody to Conserved Parts of *Plasmodium falciparum* Merozoite Surface Protein 1 in Ghanaian Children Are Not Associated with Protection from Clinical Malaria

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The 19-kDa conserved C-terminal part of the *Plasmodium falciparum* merozoite surface protein 1 ($PfMSP1_{19}$) is a malaria vaccine candidate antigen, and human antibody responses to $PfMSP1_{19}$ have been associated with protection against clinical malaria. In this longitudinal study carried out in an area of stable but seasonal malaria transmission with an estimated parasite inoculation of about 20 infective bites/year, we monitored 266 3- to 15-year-old Ghanaian children clinically and parasitologically over a period of 18 months. Blood samples were collected at the beginning of the study before the major malaria season in April and after the season in November. Using enzyme-linked immunosorbent assay, we measured antibody responses to recombinant gluthathione *S*-transferase–$PfMSP1_{19}$ fusion proteins corresponding to the Wellcome and MAD20 allelic variants in these samples. Prevalence of antibodies recognizing the Wellcome 19 construct containing both epidermal growth factor (EGF)-like motifs in Wellcome type $PfMSP1_{19}$ was about 30%. Prevalence of antibodies to constructs containing only the first EGF domain from either Wellcome or MAD20 type $PfMSP1_{19}$ was about 15%, whereas antibodies recognizing a construct containing only the second EGF domain of MAD20 type $PfMSP1_{19}$ was found in only about 4% of the donors. Neither the prevalence nor the levels of any of the antibody specificities varied significantly with season, age, or sex. Significantly, and in contrast to previous reports from other parts of West Africa, we found no evidence of an association between antibody responses to $PfMSP1_{19}$ and clinical protection against malaria.

The asexual blood stages of the *Plasmodium falciparum* parasite are responsible for the clinical manifestations of malaria, and attempts have consequently been made to identify asexual stage antigens that may be of importance in the development of protective immunity to the disease (43). One such well-characterized antigen is the *P. falciparum* merozoite surface protein 1 (PfMSP1), which is located on the surface of blood stage merozoites. It is synthesized as a 200-kDa protein during schizogony but processed into fragments with diverse molecular weights, most of which are discarded before erythrocyte invasion (30). The final processing of the C-terminal 42-kDa fragment yields a 33-kDa protein, which is shed, and a relatively conserved 19-kDa part ($PfMSP1_{19}$), which remains attached to the merozoite during erythrocyte invasion and is expressed by the parasite during the early ring stages (29). Antibodies against this fragment may block merozoite invasion of erythrocytes and also inhibit parasite multiplication inside the erythrocytes (28, 29). The objective of this study was to verify the previous finding of association between antibody responses to $PfMSP1_{19}$ and protection from clinical malaria (23) and to characterize how donor age and season influence the levels of these antibodies.

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MATERIALS AND METHODS

Study area. The study was conducted in Dodowa, a semirural town approximately 50 km northeast of Accra, Ghana. It is predominantly a subsistence farming community with a population of about 6,500. There are two rainy seasons in this area: a major rainy season from May to August, and a minor one occurring between October and November. This is followed by a relatively dry season from December to April. Malaria transmission is perennial, but is highest during or immediately after the major and minor rainy seasons (high-transmission season) and lowest during the dry season (low-transmission season). It has been estimated that individuals in Dodowa are exposed to about 20 infective bites per year, and 98% of the infections are due to *P. falciparum* (1). Dodowa can thus be described as an area of hyperendemic and seasonal malaria transmission. The transmission is stable since it does not vary considerably from year to year.

Study population and clinical surveillance. The study population consisted of a cohort of 300 schoolchildren, 3 to 15 years of age, of whom 54% were males and 46% were females. The cohort included between 13 and 37 children at each year of age. Informed parental consent was obtained after thorough explanation of all procedures involved in the study, which was approved by the Ghanaian Ministry of Health. The children selected were typed negative for sickle cell trait prior to the start of the study in April 1994. The study was completed in August 1995. During this period, the cohort was monitored clinically and parasitologically with the help of six field assistants who were resident in the town. Each child was visited once a week; during each visit, information regarding the health status in the previous week was recorded on a standard questionnaire form, and measurement of axillary temperatures was determined with a digital thermometer. Blood slide samples for detection of parasitemia were made from children with temperatures of ≥37.5°C and from children complaining of symptoms suggestive of malaria. Parents were also instructed to bring sick children to the field assistants outside the weekly scheduled visits, for recording of temperature and blood sampling by fingerprick. Any child with detectable parasitemia and fever was immediately treated with chloroquine, but for the analysis of data individuals were considered to have malaria only if (i) they reported fever and/or had a measured temperature higher of than 37.5°C and (ii) they had parasitemia of ≥5,000 parasites/μl. For the duration of the study, blood slide samples were

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Cloning and Partial Characterization of Regulated Promoters from *Lactococcus lactis* Tn*917-lacZ* Integrants with the New Promoter Probe Vector, pAK80

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Transposon Tn*917*-LTV1 was used to produce a collection of *Lactococcus lactis* strains with fusion of a promoterless *lacZ* gene to chromosomal loci. Screening 2,500 Tn*917*-LTV1 integrants revealed 222 that express β-galactosidase on plates at 30°C. Pulsed-field gel electrophoresis revealed Tn*917*-LTV1 insertions in at least 13 loci in 15 strains analyzed. Integrants in which β-galactosidase expression was regulated by temperature or pH and/or arginine concentration were isolated. In most cases, the regulation observed on plates was reproducible in liquid medium. One integrant, PA170, produces β-galactosidase at pH 5.2 but not at pH 7.0, produces more β-galactosidase at 15°C than at 30°C, and has increased β-galactosidase activity in the stationary phase. DNA fragments potentially carrying promoters from selected *Lactococcus lactis* integrants were cloned in *Escherichia coli*. A new promoter probe vector, pAK80, containing promoterless β-galactosidase genes from *Leuconostoc mesenteroides* subsp. *cremoris* and the *Lactococcus lactis* subsp. *lactis* biovar diacetylactis citrate plasmid replication region was constructed, and the lactococcal fragments were inserted. Plasmid pAK80 was capable of detecting and discriminating even weak promoters in *Lactococcus lactis*. When inserted in pAK80, the promoter cloned from PA170 displayed a regulated expression of β-galactosidase analogous to the regulation observed in PA170.

Lactococcus lactis is an important industrial microorganism used to produce a variety of cheeses and cultured milk products such as buttermilk. Genetic modification of *Lactococcus lactis* may be desirable, for example, to improve the acid production, bacteriophage resistance, or production of flavor compounds by industrially important strains. A better understanding of the regulation of gene expression in this organism and a collection of regulated promoters would facilitate the desired modifications.

Regulation of gene expression in *Lactococcus lactis* has been the subject of several studies (recently reviewed in references 11 and 36). A variety of promoter selection vectors have been developed; these vectors allow detection of promoters in *Lactococcus lactis* following insertion of DNA fragments into a polylinker preceding a promoterless reporter gene (1, 6, 22, 31, 33, 38). The function and regulation of these promoters were, in most cases, not elucidated. Several promoters from defined lactococcal genes have been shown to be regulated (9, 12, 15, 35, 39).

Accurate analysis of the regulation of gene expression is not always possible with multicopy systems. Several transposons carrying a promoterless reporter gene have been constructed and used as promoter probes in enterobacteria (3), allowing analysis of gene expression with one copy per chromosome. Youngman et al. (41) used the gram-positive *Enterococcus faecalis* transposon Tn*917* for studying the sporulation genes in *Bacillus subtilis*. Recently, we reported that Tn*917-lacZ* derivatives, including Tn*917*-LTV1 (8) (Fig. 1) and Tn*917*-TV32 (40), transpose in *Lactococcus lactis* and that the chromosome of this bacterium does not contain hot spots for Tn*917* insertions (18). Ten *Lactococcus lactis* Tn*917*-TV32 integrants that express β-galactosidase, presumably as a result of fusion of chromosomal promoters to the *lacZ* gene, were isolated (18).

In this paper, we describe the production of a collection of 222 *Lactococcus lactis* Tn*917*-LTV1 integrants that express β-galactosidase (β-Gal$^+$). Screening of integrants on plates for regulated expression of β-galactosidase was used as an initial step in identification of sites on the *Lactococcus lactis* chromosome where regulated gene expression occurs upon gene insertion. *Lactococcus* DNA adjacent to the *lacZ* end of the transposon from five Tn*917-lacZ* integrants was cloned in *Escherichia coli*. These integrants were chosen because β-galactosidase expression responds to environmental factors relevant to production processes used in dairies. A new promoter probe vector, pAK80, was constructed and shown to be more sensitive than pGKV210 (37) for promoter detection. Finally, we show that, when one of the promoters is inserted in pAK80, regulation of gene expression is analogous to the regulation observed in the original Tn*917*-LTV1 integrant.

(Preliminary results of this work were presented at the Fourth Symposium on Lactic Acid Bacteria, Noordweijkerhout, The Netherlands, September 1993, and at the ASM Fourth International Conference on Streptococcal Genetics, Santa Fe, N.Mex., May 1994.)

MATERIALS AND METHODS

Bacterial strains, culture media, reagents, and plasmids. *Lactococcus lactis* MG1363, MG1614 (14), and derivatives were routinely grown in M17 (34) containing 0.5% glucose instead of lactose at 30°C, unless otherwise specified. *E. coli* DH5α (Life Technologies, Gaithersburg, Md.) was cultured at 37°C in Luria-Bertani (LB) broth or LB agar (4). Erythromycin (ERY), chloramphenicol (CAM), ampicillin (AMP), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were purchased from Sigma Chemical Co. (St. Louis, Mo.). ERY was used for selection of pGKV210 and pAK80 derivatives in *Lactococcus lactis* at 5.0 and 1.0 μg/ml,

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Cooperation Between Antibodies and Monocytes that Inhibit In Vitro Proliferation of *Plasmodium falciparum*

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The cooperative effect between nonsensitized monocytes from normal individuals and malarial antibody in depressing parasite multiplication was investigated in an in vitro assay. The addition of purified normal monocytes to *Plasmodium falciparum* cultures in the presence of serum from immune individuals markedly inhibited the proliferation of the parasite in vitro: the parasitemia observed was about 22 times lower than that in the presence of immune serum alone. This cooperative effect was found to be effective over a wide range of monocyte/erythrocyte ratios (1:10 to 1:160) and serum concentrations (1/10 to 1/80). Immunoglobulin G extracted on protein A-Sepharose was as effective as total serum in this system. These data suggest that cooperation between nonsensitized cells and immunoglobulins could be an important effector mechanism against *P. falciparum* parasites in vivo.

The infection of humans by malaria parasites elicits an initial immune response, with specifically sensitized T cells and high titers of antibodies, which is generally unable to control infection. However, in individuals repeatedly infected over several years, a state of incomplete resistance, called premunition, appears very gradually. Despite extensive studies with laboratory models of malaria and, to a lesser extent, with humans, the mechanisms mediating protective immunity remain unknown.

The main experiments from which important information has been gained concerning the effectors of the immune response in humans are those of passive transfer. Immunoglobulin G (IgG) extracted from the serum of naturally immunized individuals caused a dramatic fall in the number of asexual parasites when injected into nonimmune recipients (11, 19). Moreover, this protection seemed to be strain independent (20). However, upon addition of serum from immune individuals to *Plasmodium falciparum* cultures, we found (unpublished data), as have others (3, 23, 29), that antibody only partially decreased the multiplication rate and sometimes increased it, but never stopped in vitro proliferation.

Since immune IgG has proved to be more effective in vivo than in vitro, its effectiveness may be related to the involvement of other components of the immune system, particularly nonsensitized cell populations. Indeed, it has been shown recently (4) that addition of immune serum together with large numbers of lymphocytes (lymphocyte/target ratio = 50:1) inhibits markedly the in vitro proliferation of *P. falciparum*. However, in this experiment the cells were isolated from malaria-sensitized individuals, and all mononuclear cell types, including lymphocytes, monocytes, and some polymorphonuclear leucocytes, were used. On the other hand, we have previously shown, using an in vitro method, that human peripheral blood monocytes are involved in the clearance of the free blood stages of *P. falciparum*, the merozoites, after being armed in vivo (17) or in vitro (16) by cytophilic IgG from immune individuals.

The present study was designed to investigate the effect of cooperation between normal monocytes and malarial antibodies on in vitro proliferation of *P. falciparum* in comparison to the effect of antibodies alone.

MATERIALS AND METHODS

Monocytes. The monocytes used in the inhibition assay were obtained from healthy individuals with no past history of malaria. Mononuclear cells isolated from heparinized blood by use of a high-density Ficoll-Hypaque gradient (2) were separated into two populations based on their ability to adhere to serum-coated plastic dishes (18). After three washes of the dishes with cold culture medium, the cell population adhering to the culture dish consisted of 90 to 95% monocytes, and the remaining cells were lymphocytes and neutrophils. Viability, measured by dye exclusion, ranged from 85 to 95%.

Sera. Sera containing malarial antibodies were collected from 10 West African adults who were selected because they had been continuously exposed to malar-

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Effector Cells Involved in Nonspecific and Antibody-Dependent Mechanisms Directed against *Plasmodium falciparum* Blood Stages In Vitro

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We have evaluated in in vitro conditions the possible cooperative effect of antimalarial antibodies with several human blood cell types. When used alone, immunoglobulin G from African adults who had reached a state of premunition against malaria was found to have no or very limited direct effect on invasion and multiplication of *P. falciparum* asexual blood stages. In contrast, these antibodies induced a marked specific inhibition of parasite growth in the presence of normal blood monocytes, and the inhibition did not appear to be strain dependent. No similar antibody-dependent cellular inhibitory effect was found using human blood polymorphonuclear leukocytes, lymphocytes, platelets, or adherent spleen cells. However, these cells could all exert in vitro some non-antibody-dependent inhibitory effect when present at high effector/target cell ratios.

In human malaria, relatively few data are available on the effectors of the immune system in subjects who have reached a state of premunition. Nevertheless, the involvement of antibodies has been clearly established in three separate in vivo experiments examining more than 30 subjects: when immune immunoglobulin G (IgG) from protected adults was injected into nonimmune children, *Plasmodium falciparum* parasitemia decreased markedly (10, 12, 19).

Once a cultivation method for *P. falciparum* asexual blood stages was established, it provided a means to modelize under in vitro conditions the mechanisms underlying the observed in vivo effect. Our previous studies in vitro did not indicate that antibodies from protected subjects had any major direct effect on the asexual blood stage cycle. Rather, these studies clearly suggested that IgG cooperates with blood monocytes in an antibody-dependent cellular cytotoxicity (ADCC) mechanism (17).

In the present study, we investigated the possible involvement of other blood and tissue cell types in ADCC by comparing the effects of these cells with those of antibodies alone. Because of the relative fragility of infected erythrocytes (RBC), classical ADCC assays could not be carried out in a reliable way. The cytotoxic effect was in fact measured indirectly by assessing in vitro parasite growth. Therefore, the assay will thereafter be called ADCI (antibody-dependent cellular inhibition).

MATERIALS AND METHODS

Sera. We studied 34 sera which presumably contained protective antibodies since they were obtained from immune African adults. The premunition state of the donors was defined on clinical and epidemiological grounds, namely, continuous exposure to malaria since childhood in the absence of any preventive measures, such as drug prophylaxis, or control measures against mosquitoes.

IgG was prepared from 16 of these sera by ion-exchange chromatography on DEAE-trisacryl (IBF) in Tris hydrochloride (pH 8.2) buffer. The IgG fraction was thereafter dialysed with RPMI, concentrated to the original serum volume, and sterilized on 0.22-μm-pore-size Millex filters. Immunofluorescence was used to check the titer of antimalarial antibodies in the IgG prepared.

Control sera and control IgG were obtained (i) from healthy French blood donors with no history of malaria, and (ii) from *P. falciparum* primary attack cases in French travellers, collected during or shortly after the attack (such individuals have high antibody titers but have no clinical protection against reinfection). Control samples were kept in similar conditions (at −20°C) and for about the same duration as the African sera.

***P. falciparum* growth inhibition assay.** We used two African strains (UPAS and NF 54) and one Thai isolate (FCPS-T23) of *P. falciparum*. The strains were cultured in RPMI medium plus 10% human serum as described (17) and were used within 2 months after thawing of the stabilates. Both NF 54 and FCPS-T23 strains retained their ability to form knob protrusions at the RBC membrane level, as shown by electron microscopic studies (P. Oliaro et al., unpublished data). Only those cultures with a high in vitro rate of proliferation were used in the inhibition assays.

The effects of patient serum, of purified IgG, of cells alone, and of cells and antibodies upon *P. falciparum* growth were assessed in 48-h cultures of *P. falciparum* without medium replacement, performed in 24-well plates containing 0.5 ml of medium per well. Unless otherwise stated, cultures were asynchronous, the initial parasitemia was 0.3 to 0.5%, and the hematocrit was 2%. Patient serum as control serum was added to RPMI medium at a concentration of 10%, and IgG was added at a final concentration corresponding to 10% of its original concentration in the donor serum, with 10% normal culture serum. The number of effector cells in each well is expressed here as a ratio with relation to total RBC and not to infected RBC, since the latter varied during the culture period. We used effector cells at ratios of 1/10 to 1/200 RBC (except for platelets, added at ratios of 1/1 to 1/40 RBC). Each plate included as controls normal human serum (or IgG from normal subjects plus 10% normal serum), patient serum alone (or IgG from patient serum plus 10% normal serum), and effector cells with normal serum (or effector cells with normal IgG plus 10% normal serum).

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